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**Early acute anti-HLA antibody-negative microvascular rejection of kidney transplants is associated with preformed IgG antibodies against diverse glomerular endothelial cell antigens**

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## **SIGNIFICANCE STATEMENT**

Antibody-mediated rejection (AMR) is associated with a poor transplant outcome. Pathogenic alloantibodies are usually directed against human leucocyte antigens (HLAs). However, the evidence of AMR in the absence of anti-HLA antibodies suggests the presence of non-anti-HLA antibodies, which are designated anti-endothelial cell antibodies (AECAs). This manuscript describes the clinicopathological profiles of kidney recipients who experienced acute rejection with microvascular inflammation within the first 3 months after transplantation in the absence of anti-HLA donor-specific antibodies. A new endothelial crossmatch, combined with transcriptomic and proteomic analyses, revealed that prior to transplantation, these patients carried unknown AECAs in their sera that specifically targeted the glomerular microvascular endothelium. An assessment of these unknown AECAs with potential deleterious effects may provide important diagnostic tools to prevent AMR.

## ABSTRACT

**Background:** Although the majority of antibody-mediated rejections (AMRs) of renal allografts are due to anti-HLA antibodies, non-anti-HLA antibodies have also been postulated to contribute. The occurrence of non-anti-HLA-associated AMRs remains associated with unresolved diagnostic and therapeutic issues.

**Methods:** Through a nationwide study, we identified kidney transplant recipients (KTRs) without anti-HLA donor-specific antibodies who experienced acute graft dysfunction within the first 3 months after transplantation and showed severe microvascular injury in a biopsy (called acute microvascular rejection, AMVR) to better understand the pathological mechanisms of these rejections.

**Results:** A highly selected cohort of 38 patients with AMVR was identified. AMVR occurred at  $22.0 \pm 26.2$  days post-transplantation. Biopsies revealed intense microvascular inflammation with a mean Banff glomerulitis and peritubular capillaritis (g+ptc) score of  $3.9 \pm 0.25$ , vasculitis in 60.5% of cases, interstitial hemorrhages in 31.6% of cases and thrombotic microangiopathy in 15.8% of cases. Compared to a control group of stable KTRs, patients with AMVR did not show a significant increase in angiotensin type 1 receptor (AT1R), endothelin-1 type A (ETAR) or natural polyreactive antibodies (NABs). Using the threshold of 10 IU/mL, 26% of the tested AMVR were found positive for AT1R Abs. A homemade endothelial crossmatch identified a common IgG response that was specifically directed against constitutively expressed antigens of microvascular glomerular cells in patients with AMVR. Using a combination of transcriptomic and proteomic approaches, we identified new targets of non-HLA antibodies with little redundancy among individuals.

**Conclusions:** Based on our results, preformed IgG antibodies targeting non-HLA antigens expressed on glomerular endothelial cells are associated with early AMVR and *in vitro* cell-

based assays are needed to improve risk assessments before transplantation.

## INTRODUCTION

Despite the development of potent immunosuppressive regimens, antibody-mediated rejection (AMR) remains a significant hurdle to long-term organ acceptance. Although histological findings suggestive of AMR (i.e., microvascular inflammation) usually indicate an anti-human leukocyte antigen (HLA)-mediated injury, a subset of patients develop these lesions in the absence of detectable anti-HLA donor-specific antibodies (DSAs). The potential involvement of non-HLA antibodies (Abs) is mentioned in the current Banff classification, which requires the presence of “serological evidence of DSAs against HLA or other antigens”. However, in the absence of other, clearly defined antigens, the assumption that acute rejections with significant microvascular inflammation (called AMVRs hereafter, for acute microvascular rejections) are true AMRs remains hypothetical. In addition, although this issue is of utmost importance for treatment decisions, a clear indication that the observed graft injury is induced by Abs may be difficult to obtain.

These particular types of immune injuries are presumed to be due to Abs that react with non-HLA antigens expressed on endothelial cells (ECs). These Abs might be alloantibodies directed against non-HLA polymorphic antigens that differ between the recipient and donor or autoantibodies that recognize self-antigens following a disruption of self-tolerance<sup>1</sup>. The identification and characterization of pathogenic anti-endothelial cell Abs (AECAs) would improve our understanding of the mechanisms involved in AMRs and would enable the development of new tools for patient monitoring. Several hurdles hamper the identification of these AECAs. First, the development of acute renal dysfunction with histological lesions suggestive of AMR in the absence of anti-HLA DSAs is a relatively rare event. Consequently, previous studies that aimed to identify AECAs often included patients with heterogeneous



clinical presentations ranging from hyperacute rejection<sup>2-4</sup> to chronic allograft dysfunction<sup>4,5</sup> or patients with a positive EC crossmatch independent of any clinical presentation<sup>6</sup>. Second, the identification of deleterious non-HLA Abs is particularly difficult to achieve in long-term patients, as a broad autoantibody response develops over time after transplantation<sup>7,8</sup>.

We aimed to study a highly selected cohort of patients with a homogeneous clinical and pathological presentation of AMVR without anti-HLA DSAs during the first 3 months post-transplantation to overcome these challenges. We reasoned that early AMVR would likely be caused by preformed AECAs, facilitating their identification in pretransplant serum samples. We report here the clinicopathological description of this cohort and our efforts to identify the pathogenic AECAs.

## **METHODS**

### ***Patients***

Kidney transplant recipients (KTRs) were identified through a nationwide survey aimed at identifying suspected cases of early AMVRs of renal allografts in the absence of anti-HLA DSAs. Inclusion criteria were a first transplantation or retransplantation, a deceased or living donor, acute dysfunction or delayed graft function occurring within the first 3 months post-transplantation, histological features of microvascular inflammation with a glomerulitis and peritubular capillaritis (g+ptc) score greater than or equal to 3 according to the Banff classification, and the absence of historical or current anti-HLA DSA (A/B/Cw/DR/DQ/DP), as assessed using a Luminex® single-antigen bead assay (all mean fluorescence intensities (MFIs)<500). All biopsies were centrally reassessed, and the absence of anti-HLA DSAs was also centrally confirmed (see the [Supplementary Methods](#) for details).

For the case-control histological study ([Figure 1](#)), a control group of 20 KTRs with early full-blown AMR who presented with anti-HLA DSAs in the first three months was identified. The patients were matched for age, gender, time of transplantation and immunosuppressive regimen at transplantation.

For the case-control biological study ([Figure 1](#)), a second control group of 10 highly stable patients (i.e., no rejection during the first year) was identified. Patients in this control group were also matched to patients in the AMVR group for age, gender, time of transplantation and immunosuppressive regimen at transplantation.

### ***Non-HLA antibody detection***

Methods for the detection of non-HLA Abs, including anti-MICA, anti-AT1R, anti-ETAR, natural Abs and Abs against a panel of 62 non-HLA antigens, in patients' sera are described in detail in the [Supplementary Methods](#).

### ***EC crossmatch***

Different types of ECs were incubated with patients' serum samples, and IgG fixation was detected using flow cytometry. A comparative analysis of the reactivity of the patient's serum was performed on parallel crossmatches using primary cultures of non-donor-specific arterial ECs and the immortalized human glomerular microvascular EC line CiGEnC (see the [Supplementary Methods](#) for details).

### ***RNA sequencing (RNAseq) and protein array***

RNAseq was performed to assess the differences in the transcriptomes between microvascular and macrovascular ECs. Patients' serum samples were applied to a protein

array to assess the seroreactivity of stable KTRs and patients with AMVR (see the [Supplementary Methods](#) for details).

### ***Statistics***

The results are presented as the means $\pm$ SD for continuous variables, unless specified otherwise. Frequencies of categorical variables are presented as numbers and percentages. Analyses were performed with GraphPad Prism software (version 5.00; GraphPad Software, San Diego, CA). For statistical comparisons of the clinical data between two groups, we used unpaired two-tailed t tests and a chi-square test. For statistical comparisons of the *in vitro* data, we used nonparametric tests. P values<0.05 were considered significant.

A detailed description of the statistical methods used to analyze the protein array and RNAseq data is provided in the [Supplementary Methods](#).

## **RESULTS**

### ***Clinicopathological description***

A nationwide survey identified 51 KTRs (from 21 centers) with suspected early AMVR in the absence of anti-HLA DSAs (DSA- AMVR). After a central reassessment of anti-HLA DSAs (AC) and a central histological analysis (MR and JPD), the final cohort included 38 patients with confirmed early acute DSA- AMVR ([Figure 1](#)).

Patients were 43.0 $\pm$ 14.3 years of age ([Table 1](#)). Ten of the 38 patients with AMVR (26.3%) received a second (n=9) or a third (n=1) transplant ([Table 1](#)). Nineteen patients with AMVR (50%) presented non-DSA anti-HLA antibodies at transplant. Comparing retransplantations to first transplantations within the AMVR group, no difference in the anti-HLA sensitization,

neither to class I (5/28 vs. 4/10,  $P=0.21$ ) nor to class II HLA molecules (6/28 vs. 4/10,  $P=0.40$ ), was observed.

AMVR was diagnosed at a mean time of  $11.2\pm 1.7$  days for the 18 patients still requiring hemodialysis. For the other 20 patients, AMVR was diagnosed based on an increase in the serum creatinine level from  $275\pm 187$   $\mu\text{mol/L}$  at  $15.7\pm 21.4$  days to  $417\pm 276$   $\mu\text{mol/L}$  at  $31.8\pm 7.3$  days post-transplantation.

The AMVR treatment was heterogeneous. However, rituximab was administered to 31.6% of patients, plasmapheresis to 65.8% and intravenous immunoglobulins to 47.4%, suggesting that the patients were considered as having AMR.

A comparison of patients with DSA- AMVR with matched patients with DSA+ AMR ([Table 1](#)) revealed that patients with DSA- AMVR displayed similar graft function at the rejection diagnosis ( $417\pm 276$   $\mu\text{mol/L}$  vs  $298\pm 229$   $\mu\text{mol/L}$ ,  $P=0.11$ ), more severe graft dysfunction at 3 months ( $161\pm 59$   $\mu\text{mol/L}$  vs  $129\pm 55$   $\mu\text{mol/L}$ ,  $P=0.0098$ ), and similar graft function at the last follow-up ( $P=0.23$ ). Consistent with severe graft injury, proteinuria was common in both groups, and after a similar follow-up period, the proteinuria in the AMVR cohort was similar to the AMR cohort ( $1.27\pm 1.7$  g/g vs  $1.0\pm 1.4$  g/g,  $P=0.44$ ).

The central histological reading of the patients with DSA- AMVR showed severe microvascular inflammation, with a mean g+ptc score of  $3.9\pm 0.25$  ([Figure 2A and 2B](#)), and severe endothelial/vascular injury ([Figure 2C-H](#)). Vasculitis was present in 60.5% of cases, and thrombotic microangiopathy and interstitial hemorrhages were observed in 15.8% and 31.6% of cases, respectively ([Table 2](#)).

Compared to DSA+ AMR biopsies, DSA- AMVR biopsies exhibited more severe endothelial/vascular injury, with significantly more v lesions ( $1.3\pm 1.1$  vs  $0.3\pm 0.8$ ,  $P=0.0003$ ), a greater number of cases with vasculitis lesions (60.5% vs 15%,  $P=0.001$ ) and numerically

more thrombotic microangiopathy (15.8% vs 0%,  $P=0.08$ ) ([Table 2](#)). Compared to patients with AMR, patients in the AMVR group showed significantly more interstitial infiltrates. Overall, T cell-mediated rejection defined according to the Banff classification was not significantly different between the two groups (31.5% vs 10.0%,  $P=0.18$ ).

### ***Assessment of known AECAs***

The presence of previously proposed AECAs<sup>9, 10</sup> was assessed in available serum samples collected at the time of transplant (day 0), corresponding to a mean time of  $22.0 \pm 26.2$  days prior to the AMVR diagnosis, in 23 patients with early AMVR and 10 stable KTRs used as controls ([Supplementary Table 1](#)).

Anti-MICA Abs were detected in only two patients with AMVR.

Titers of angiotensin type 1 receptor (AT1R) and endothelin-1 type A (ETAR) Abs were similar in both groups ([Figure 3A](#)). Regarding AT1R Abs, we did not observe any positivity in the AMVR group or in the stable group ([Figure 3A](#)) using the threshold of 17 UI/mL proposed by Hönger et al.<sup>11</sup>. When the positive threshold of 10 UI/mL proposed by Dragun et al.<sup>2</sup> was used, 6/23 patients with AMVR (26%) were positive for AT1R Abs compared to no patients (0/10, 0%) in the stable group ( $P=0.14$ , chi-square test). However, we observed a good correlation between ETAR and AT1R Ab titers, with an  $r^2$  greater than 0.8 ( $P<0.0001$ ), suggesting a spread of the Ab response toward more autoreactivity ([Figure 3B](#)).

IgG natural polyreactive antibody (NAb) levels were assessed in serum samples from patients with AMVR and control subjects using two separate methods. No difference in IgG NAbs was observed between the two groups with either method ([Figure 3C](#)). However, as reported in [Figure 3D](#), the level of IgG NAbs measured using an ELISA was significantly

correlated with the level of anti-ETAR Abs, supporting the view of a broad autoimmune component.

Sera were also tested against a panel of 62 non-HLA antigens ([Figure 3E](#)). At the time of transplant, 19/23 (83%) patients with AMVR tested positive for at least one of the non-HLA antigens examined, whereas no stable patients reached the positivity threshold. Sixteen of the 62 antigens were positive in at least one patient with AMVR. A total of 45 antigens were found positive, with a maximum of 8 patients with AMVR exhibiting positivity for protein kinase C $\zeta$  (PKC $\zeta$ ). Overall, no antigen appeared to be a positive target in the majority of patients with AMVR.

### ***EC crossmatch***

Since no AECA candidate explained the majority of AMVR cases, we developed an EC crossmatch assay to assess serum reactivity to human microvascular glomerular ECs<sup>12</sup>. Two EC types were used as cellular targets: non-donor-specific primary cultures of human vascular arterial ECs and the CiGEnC line, an established thermosensitive conditionally immortalized cell line that allows cells to differentiate into glomerular microvascular ECs at 37°C with a preserved endothelial phenotype. The CiGEnC phenotype observed after differentiation is shown in [Supplementary Figure 1](#). As ECs express class I and class II HLA antigens, this analysis was restricted to patients with AMVR, stable KTRs or healthy volunteers with no circulating anti-HLA Abs to avoid any HLA-dependent cell reactivity. Strikingly, the seroreactivity against glomerular ECs was significantly increased in sera from patients with AMVR ([Figure 4A](#)), whereas limited reactivity was observed in healthy volunteers (n=6) or stable KTRs (n=10). Seroreactivity against non-HLA antigens was only due to IgG, as no IgM reactivity was observed (data not shown). This IgG reactivity was present

on day 0 (**Figure 4B**) and persisted to the time of rejection. Serial titration of positive sera revealed high Ab titers (**Figure 4C**).

Crossmatches were also performed in resting ECs and after TNF- $\alpha$  and IFN- $\gamma$  stimulation to better characterize the seroreactivity of patients with AMVR. The stimulated status of the CiGENCs after cytokine treatment was controlled by the upregulation of the HLA molecules (**Supplementary Figure 2**). In healthy controls, even after cell activation, no significant reactivity toward glomerular ECs was observed, compared to 89% positivity in patients with AMVR. Interestingly, the high-level seroreactivity observed in patients with AMVR did not depend on inflammation (**Figure 4D**), suggesting that the antigen targets are basally expressed on CiGENCs and are not regulated by TNF- $\alpha$  or IFN- $\gamma$ . Moreover, no significant reactivity was observed using primary cultures of human macrovascular ECs as targets, even after cell stimulation (**Figure 4E**), or using human renal epithelial cells as targets. Finally, patients with AMVR exhibited higher seroreactivity toward fully differentiated glomerular ECs than against undifferentiated ECs (**Figure 4F**).

Based on these results, the targeted antigens are selectively and constitutively expressed on the surface of glomerular ECs.

### ***Integrated cDNA-protein array analyses of glomerular EC-specific immunogenicity***

As AMVR seroreactivity specifically targeted glomerular ECs but not macrovascular ECs, we first assessed the differences in the transcriptomic profiles of these two cell types to identify antigens restricted to microvascular ECs (**Figure 5A**).

Unsupervised hierarchical clustering of mRNA expression patterns correctly classified the microvascular and macrovascular ECs (**Figure 5A**), suggesting that microvascular glomerular ECs have a distinct transcriptomic profile. Next, read count normalizations and group

comparisons were performed using three independent and complementary methods that identified 3427 differentially expressed transcripts in the two cell types (**Supplementary Figure 3**), including 2195 genes that were significantly overexpressed in microvascular ECs compared with macrovascular ECs (available online, [www.ebi.ac.uk/fg/annotare](http://www.ebi.ac.uk/fg/annotare) E-MTAB-7003).

We then used a protein array platform to assess the reactivity of serum samples collected immediately before transplantation from 20 patients with early AMVR and 10 patients who remained stable over the first year after transplant to approximately 9375 antigens. An evaluation of the average signals for the anti-human IgG revealed values that were within the expected ranges and were consistent across the arrays, indicating the good quality of the samples from both groups. An unsupervised principal component analysis (PCA) revealed a clear separation of sera from patients with AMVR from sera from stable patients (**Figure 5B**) suggesting that the global seroreactivity profile of patients with AMVR was different.

Following normalization, individual antigens from protein arrays were ranked according to the frequency of reactivity of AMVR sera compared to control sera. Antigen-specific responses must have been more prevalent in the sera from patients with AMVR than in sera from the stable patients to be considered an antigen of interest, thus possibly representing shared immunogenic events targeting microvascular ECs. Compared with sera from stable patients, sera from patients with AMVR preferentially reacted with 136 of 9375 antigens (unadjusted  $P < 0.05$ , **Supplementary Table 2**), but substantial variability was observed among individuals, as illustrated in **Figure 5C**.

We next performed an integrated analysis (**Figure 1**) combining the serological responses of the patients with AMVR and stable KTRs to the microvascular EC-specific mRNA expression profiles, with the aim of identifying non-HLA Abs in patients with AMVR that



target proteins specifically expressed by glomerular microvascular ECs. This strategy allowed us to identify a list of 857 matches of immunogenic antigens and overexpressed genes in microvascular ECs ([Figure 1](#)).

Because seroreactivity was highly variable among patients with AMVR, we rank-ordered the 857 potential targets using a previously described method<sup>7</sup> that calculates a global score for each candidate by including the frequency of seroreactivity in patients with AMVR compared with stable patients and the relative strength of the reactivity. Thus, numerous unidentified AECAs are present in patients with AMVR, but not in stable patients ([Table 3](#)).

Finally, 4 genes identified using our integrated RNAseq-protein array analysis were selected from Table 3 and validated at the mRNA and protein levels in microvascular ECs. As shown in [Supplementary Figure 4](#), the 4 genes, bone morphogenetic protein receptor type 1A (BMPRI1A), ephrin type-B receptor 6 (EPHB6), leiomodulin-1 (LMOD1) and myelin basic protein (MBP), are expressed in the endothelial crossmatch target cells.

## DISCUSSION

The concept that AMR may arise in the absence of anti-HLA DSA is universally accepted<sup>13</sup>. This particular type of rejection is still improperly diagnosed, primarily because of the unknown specificity of the non-HLA Abs associated with its manifestation. Its clinical course and impact on the transplant outcome are also largely unknown. In an effort to better understand this complication, we studied a cohort of highly selected KTRs who experienced an AMR that was likely triggered by non-HLA DSAs.

In addition to circulating Abs, C4d deposition in peritubular capillaries is considered the best surrogate of antibody-induced injury, even if this marker is not always detected in

patients with conventional AMR<sup>14, 15</sup> or in the context of suspected AECA-related AMR<sup>3, 16</sup>. In the absence of a consensus definition, we restricted our inclusion criteria to patients with significant microvascular inflammation. In addition, we selected KTRs experiencing acute rejection within the first three months after transplantation, resulting presumably from preformed Abs. These criteria allowed us to identify patients with a homogeneous clinical and pathological presentation. In addition to a severe clinical phenotype, the histological assessment revealed a dramatic involvement of the vascular wall with an unusual frequency of vasculitis lesions, thrombotic microangiopathy and interstitial hemorrhages. Long-term follow-up of these patients revealed allograft dysfunction and glomerular proteinuria that were also consistent with an antibody-mediated immune injury.

Numerous AECA have been reported in the last decade<sup>9</sup>. Unlike other targets, AT1R and ETAR are well-established effectors in autoimmune diseases affecting the macro- and microvasculature. AT1R agonistic antibodies have been associated with preeclampsia, malignant/refractory hypertension, and primary aldosteronism<sup>17</sup>. ETAR agonistic antibodies have been associated with systemic sclerosis and systemic lupus erythematosus associated with pulmonary hypertension<sup>17</sup>. Importantly, adoptive transfer experiments<sup>18</sup> and pharmacological inhibition in animal models<sup>19</sup> supported their pathogenic effects. In transplantation, the seminal work by Dragun et al. reported not only the association of AT1R Abs with refractory vascular rejection, but also their potential pathogenic effects, demonstrated by the transfer of AT1R Abs to a rat model of kidney transplantation<sup>2</sup>. Recent studies also suggest the association of AT1R with histological features of ABMR in indication renal allograft biopsies<sup>20</sup>. In the present study, we focused on anti-AT1R<sup>2</sup>, anti-ETAR<sup>21, 22</sup> and NAb<sup>23, 24</sup>. While none of these candidates clearly identified our patients with AMVR compared to stable KTRs, the more surprising result was that they were all correlated with

each other. Indeed, we identified a strong correlation ( $r^2=0.82$ ) between anti-AT1R and anti-ETAR Abs, a finding that was also reported previously in the context of heart<sup>21</sup> and renal transplantation<sup>10</sup>. This observation supports the hypothesis that a broad autoimmune response may occur in some patients. Consistent with this hypothesis, Butte et al. previously identified an autoantibody signature in patients with renal insufficiency compared to controls, thus suggesting that end-stage renal damage may release proteins that are not otherwise recognized as self-antigens, leading to an adaptive humoral response<sup>25</sup>. In addition, a longitudinal analysis of the Ab responses of pre-transplantation and post-transplantation sera using a protein array revealed a significant enrichment of the Ab response against kidney compartments, again suggesting that chronic organ damage induces a broad autoantibody response<sup>7</sup>. Interestingly, 26% of our AMVR cases occurred in retransplanted patients. Thus, AECAs might develop during a previous transplantation. Consistent with this hypothesis, using protein arrays, Li and al. revealed that, in addition to HLA sensitization, kidney transplantation is associated with an enrichment of a specific antibody response against different kidney compartments suggesting that non-anti-HLA Abs might develop in transplant recipients<sup>26</sup>. Further studies are needed to determine whether this autoimmune response observed in patients with end-stage renal disease and transplant recipients is due to the release of self-antigens by the damaged organ or to a systemic B cell deregulation. In this regard, our observation that the global Ab response before transplantation clearly distinguished sera from patients with AMVR from sera from stable patients supports the hypothesis of systemic B cell deregulation. More recently, an association between endothelial crossmatch positivity and AT1R Abs has also been reported<sup>27</sup>. However, in view of the increased autoimmunity observed in some patients, this association does not prove causation. Indeed, the findings reported by Dinavahi et al.<sup>28</sup> and

Porcheray et al.<sup>8</sup> that autoimmune profiles induced by transplantation are unique to each individual patient also suggest that this response potentially results from systemic B cell deregulation rather than a response to potential cryptic epitopes unmasked during chronic renal injury.

Surprisingly, our assessment of AT1R Abs revealed the paucity of highly positive sera (>17 IU/mL) for AT1R Abs compared to other published studies of renal transplantation. The small sample size and the highly selected cohort are certainly possible explanations for the lack of patients with high levels of AT1R Abs. Nevertheless, the cut-off for AT1R Ab positivity remains controversial and several studies used a threshold of 10 IU/mL<sup>29</sup>, while others used 15 IU/mL<sup>5</sup> or even 17 IU/mL<sup>11</sup>. Using a threshold of 10 IU/mL, Giral et al. reported that 47% of patients displayed AT1R Ab positivity before transplant<sup>29</sup>, while Taniguchi et al. reported that 17% of patients displayed AT1R Ab positivity before transplant using a threshold of 15 IU/mL<sup>5</sup>. In our study, using a threshold of 10 IU/mL, 6 of 23 the evaluated patients with AMVR (26%) would have been considered positive for AT1R Abs, supporting the potential role of AT1R Abs in AMVR occurrence.

We also evaluated the seroreactivity to a panel of 62 non-HLA antigens using two single-antigen flow bead assays. Although no antigen appeared to be a positive target in the majority of patients with AMVR, 8/23 patients with AMVR presented Abs against PKC $\zeta$ , which has been previously associated with acute rejection and graft loss after kidney transplantation<sup>30</sup>. The assessment of the seroreactivity to the 62 non-HLA antigens (Figure 3F) facilitated the identification of at least one antigen in 19/23 patients with AMVR, while none of the control cases exhibited a positive result, revealing the relatively good discriminative capacity of this approach. However, this experiment also confirmed the broad and variable reactivity among individuals. Considering the highly variable serum reactivity to

non-HLA endothelial antigens, our observation that IgG reactivity toward glomerular endothelial cells predicts non-HLA Ab-induced AMRs shares some similarities with historical analyses showing an increased rejection risk associated with high Panel Reactive Antibody values, prior to the use of sensitive bead assays to more specifically identify anti-HLA DSAs. In this respect, if the 62 non-HLA antigen panel used in the present study was complemented with new candidate antigens, we may be able to improve our understanding of the underlying mechanisms and identify the culprits.

Although this “candidate gene” approach did not identify irrefutable candidates, our crossmatch assay identified preformed IgGs targeting antigens that are constitutively expressed on glomerular ECs in a compartment-specific fashion. No response or a minimal response to macrovascular cells, epithelial cells and smooth muscle cells was detected (data not shown). This reactivity was highly specific to patients with AMVR, thus supporting our primary hypothesis that AMVR cases are true AMRs. Notably, an increase in IgG binding was not observed between the day of transplantation and the day of rejection (Figure 4B), suggesting that non-HLA AECAs are preformed Abs. Figure 4B even shows a small reduction in the IgG binding when comparing “day 0” and “at rejection” suggesting that circulating Abs may bind to the graft microvessels after transplantation, similar to anti-HLA DSAs<sup>31</sup>.

We observed a greater heterogeneity in the results of crossmatches that used microvascular ECs compared to macrovascular ECs. The heterogeneous results observed with the microvascular CiGEnCs are consistent with our results suggesting that each patient has peculiar AECAs and may be due to various titers of the AECAs. The relative homogeneity observed when using the macrovascular ECs as targets suggests that the KTRs have no antibodies targeting the macrovascular endothelium. Even though our crossmatch analysis did not detect IgG binding to macrovascular ECs isolated from unused pieces of artery taken

from organ donors before kidney transplantation, we cannot exclude the presence, in kidney recipients, of other AECAs targeting antigens that are not strictly specific to the microvascular endothelium. However, the specific response to microvascular renal ECs may provide some clues regarding the still unexplained observation that auto-Abs targeting ECs have no pathogenic consequences in non-transplanted patients<sup>32</sup>. In addition, their specificity toward the microvascular endothelium of the graft organ may explain why their pathogenicity is confined to the graft. This confinement to the graft of the pathogenic consequences of AECAs may suggest that the pathogenicity of AECAs first requires an initiating injury (i.e. ischemia/reperfusion injury, anti-HLA-mediated allo-immune injury, etc ...). For example, our previous study that assessed the role of natural antibodies suggested some additive effect between anti-HLA DSA and natural Abs<sup>33</sup>, a finding that was also observed for anti-HLA DSA and AT1R Abs<sup>5, 20, 27</sup>, thus suggesting that non anti-HLA autoantibodies have the potential to amplify microcirculation injury caused by alloantibodies in antibody-mediated transplant rejection<sup>32, 34</sup>.

We used non-donor-specific ECs in our crossmatch assays. However, previously identified non-anti-HLA AECAs with suspected deleterious effects on the renal allograft, such as AT1R or ETAR or natural antibodies, which are considered autoantibodies, have not been confirmed to be donor-specific allo-Abs<sup>32</sup>.

Our transcriptomic analysis revealed substantial differences in transcriptomic profiles between macrovascular and microvascular ECs, suggesting that the endothelial crossmatch performance will be highly dependent on the endothelial cell that is used as the target. The cell-based assays that have been developed to date have used various endothelial cells (HUVECs<sup>35</sup>, primary cultures of macrovascular arterial endothelial cells<sup>36</sup>, and circulating endothelial progenitors<sup>37</sup>) but have never used the endothelial cells that are actually the

target cells during the pathological process (i.e., the renal microvascular ECs). To the best of our knowledge, our EC crossmatch assay is the first to use the target cells of the pathogenic Abs in KTRs. Of course, renal microvascular endothelial cells cannot realistically be derived from every donor. Therefore, the availability of the CiGEnCs may facilitate the development of cell-based assays with a good capability of detecting non-anti-HLA AECAs.

In an effort to identify the culprits, we profiled the global IgG Ab responses in patients AMVR and compared them to controls using protein arrays. The two main conclusions of this “antibodyome-wide” approach were that the global antibodyome correctly classified patients with AMVR, but no single specific Ab explained the disease, although several Abs that emerged from our combined analysis of transcriptomic and proteomic data have been already reported in the context of autoimmune diseases ([Supplementary Discussion](#)). The protein array we used in the present study was not customized to contain endothelia-specific antigens and/or the whole spectrum of glomerular antigenic molecules, which may have led to false negative or false positive results. Nevertheless, our combined transcriptomic and proteomic approach identified new potential targets, and the expression of several of these targets in the glomerular endothelial cells was validated ([Supplementary Figure 4](#)), thus confirming the rationale of our approach. Finally, patients suffering from non-HLA Ab-induced AMRs exhibit profound alterations of their seroreactivity, but with little redundancy, and some of their Abs are able to bind to glomerular cells. Altogether, our observations complement the aforementioned literature and suggest that an attempt to identify a common Ab that may explain the entire spectrum of disease may not succeed.

If a cell-based assay designed to detect AECAs in patient serum is an appealing strategy to circumvent the large individual variability in AECA specificities, it remains a challenging technique for several reasons, such as the variability in cell quality and surface antigen

expression, the need for cell culture expertise, and the inability to test high PRA sera. The risk of cell variability is limited by the use of a well-phenotyped cell line as opposed to primary cultures of ECs or purified circulating endothelial progenitors. Our robust endothelial phenotyping approach facilitates a longitudinal assessment of the stability of the cell line over time. Finally, AECA detection must be feasible even in highly sensitized patients. As a human cell line, CiGenCs express class I and class II HLA molecules that may lead to a positive endothelial cell crossmatch due to the presence of anti-HLA antibodies in sensitized recipients. The CRISPR/Cas9 technology will be used to delete HLA molecules from the CiGenC line and establish an endothelial crossmatch that could be useful as a screening test for AECA assessment, even in highly sensitized patients. Finally, the observed binding of AECAs to the CiGenCs may enable the more precise identification of the antigenic targets, thus facilitating the refinement of the existing solid phase assays for AECA identification. Expectedly, the development of a cell-based assay using a single cell line would not account for the potential donor genetic heterogeneity and would be more suitable for assessment of auto-antibodies that recognize public antigens following a disruption of self-tolerance, as opposed to allo-antibodies targeting non-HLA polymorphic antigens. A test that could address the tremendous inter-individual variability in terms of auto-antibody response could constitute a relevant companion test that could screen for the presence of circulating AECA in conjunction with the 'candidate gene' approach.

In conclusion, we addressed the challenging problem of AMR in the absence of anti-HLA Abs in an original way by identifying a highly selected cohort of patients who likely suffered from this unusual and difficult-to-diagnose entity. Previously identified non-HLA Abs failed to differentiate patients with AMVR from stable patients, but an innovative EC crossmatch identified a universal IgG reactivity to microvascular glomerular ECs. An in-depth integrated



analysis of transcriptomic and proteomic data revealed a large Ab response mediated by deregulation with little redundancy among individuals. Based on our results, *in vitro* cell-based assays are needed to assess the presence of EC Abs with a potential deleterious effect after transplantation.

## **AUTHORS' CONTRIBUTIONS**

M.D. and D.A. designed the study, analyzed the data, created the figures and drafted the paper. S.C.S. provided the cells and reviewed and approved the manuscript. B.L. and N.C. analyzed the data and created the figures. C.B., O.A., S.P., A.C. and S.B.S. performed the experiments. B.C. analyzed the data and created the figures. M.R. and J.P.D.V.H. read the renal biopsies. E.Z. analyzed the data and revised the paper. C.L. and J.L.T. revised the paper. P.G., M.G., O.T., N.A., A.H., M.H., M.M., C.M., S.C., N.K., J.S., P.F.W., D.D., C.G., M.L., V.V., J.R., P.M., D.B. and A.L.L. participated in selecting the cohort of patients with AMVR and collected serum samples from the patients with AMVR.

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## **DISCLOSURES**

None

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**Supplementary Figure 1:** CiGENCs acquire an endothelial phenotype after 7 days of culture at 37°C.

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**Supplementary Figure 3:** Venn diagram illustrating the number of differentially expressed genes between microvascular ECs and macrovascular ECs, according to three statistical methods.

**Supplementary Figure 4:** Validation of antigen expression in micro and macrovascular ECs.

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**Table 1: Patient demographics**

Variables	AMVR without anti-HLA DSAs, N=38	AMVR with anti-HLA DSAs, N=20	P
<b>Recipient characteristics</b>			
Male, n (%)	25 (65.8)	13 (65.0)	1.00
Age at transplantation, mean±SD, yrs	43.0±14.3	50.4±15.9	0.11
Cause of end-stage renal disease, n (%)			
Glomerulonephritis	10 (26.3)	4 (20.0)	0.75
Diabetes	6 (15.8)	5 (25.0)	0.49
Cystic/hereditary/congenital	7 (18.4)	3 (15.0)	1.00
Secondary glomerulonephritis	3 (7.9)	2 (10.0)	1.00
Hypertension	2 (5.3)	0 (0.0)	0.54
Interstitial nephritis	3 (7.9)	2 (10.0)	1.00
Miscellaneous conditions	2 (5.4)	3 (15.0)	0.33
Uncertain etiology	5 (13.2)	1 (5.0)	0.65
Duration of dialysis before transplantation, mean±SD, yrs	3.9±4.4	4.8±4.9	0.44
Previous transplantation, n (%)	10 (26.3)	3 (15.0)	0.51
<b>Transplant variables</b>			
Donor age, mean±SD, yrs	50.4±12.6	52.3±17.4	0.93
Deceased donor, n (%)	28 (73.7)	17 (85.0)	0.51
Male donor, n (%)	17 (44.7)	8 (40.0)	0.79
Cold ischemia time, mean±SD, hrs	15.9±10.4	20.5±9.7	0.13
Preformed anti-HLA Abs with an MFI>500, n (%)	19 (50.0)	20 (100.0)	<0.0001
Delayed graft function, n (%)	18 (47.3)	7 (35.0)	0.41
Number of post-transplant hemodialysis session, mean±SD	2.5±4.2	2.4±2.9	0.39
<b>Immunosuppressive protocol</b>			
Induction therapy, n (%)	38 (100.0)	19 (95.0)	0.34
Basiliximab/Thymoglobuline®, n (%)	33 (86.8)/5 (13.2)	14 (75.0)/5 (25.0)	0.28
Calcineurin inhibitor-based therapy, n (%)	37 (97.4)	20 (100.0)	1.0
Cyclosporine/Tacrolimus, n (%)	11 (28.9)/26 (68.4)	3 (15.0)/17 (85.0)	0.34
Purine synthesis inhibitor, n (%)	37 (93.9)	19 (95.0)	0.35
mTOR inhibitor, n (%)	0 (0.0)	1 (5.0)	0.35
Steroid, n (%)	37 (97.4)	20 (100.0)	1.0
<b>Acute rejection description</b>			
Best serum creatinine level before AMVR, mean±SD, µmol/L	275±187	195±137	0.15
Best serum creatinine level before AMVR, mean±SD, days	15.7±21.4	8.5±8.2	0.64
AMVR diagnosis, mean±SD, days	22.0±26.2	15.9±13.5	0.92
Serum creatinine level at rejection, mean±SD, µmol/L	417±276	298±229	0.11
Patients on dialysis at time of rejection	8 (21.1)	1 (0.05)	0.14
<b>Acute rejection treatment</b>			
Steroid, n (%)	35 (92.1)	19 (95.0)	1.00
Thymoglobuline®, n (%)	10 (26.0)	2 (10.0)	0.19
Rituximab, n (%)	12 (31.6)	10 (50.0)	0.25
Plasmapheresis, n (%)	25 (65.8)	15 (75.0)	0.56
IGIV, n (%)	18 (47.4)	17 (85.0)	0.01
<b>Follow-up</b>			
Serum creatinine level at 3 months post-Tx, mean±SD, µmol/L	161±59	129±55	0.0098
Serum creatinine level at 12 months post-Tx, mean±SD, µmol/L	145±53	125±41	0.08
Mean follow-up, mean±SD, yrs	4.3±3.0	3.5±2.7	0.25
Serum creatinine level at the last follow-up, mean±SD, µmol/L	169±97	136±76	0.23
Proteinuria <sup>a</sup> at the last follow-up, mean±SD, g/g creatinine	1.27±1.7 (n=20)	1.0±1.4 (n=18)	0.44
Patient survival at the last follow-up, n (%)	37 (97.3)	18 (90.0)	0.12
Graft survival at the last follow-up, n (%)	29 (76.3)	19 (95.0)	0.51

<sup>a</sup> in patients with a follow-up >1 yr

**Table 2:** Description of the histological findings

Histological lesions	AMVR without anti-HLA DSAs, N=38	AMVR with anti- HLA DSAs, N=20	P
<b>Glomerulitis (g)</b>			
% with a g score>0	38 (100.0%)	18 (90.0%)	0.11
g score, mean±SD	2.1±0.8	1.7±0.9	0.18
<b>Peritubular capillaritis (ptc)</b>			
% with a ptc score>0	36 (94.7%)	19 (95.0)	1.0
ptc score, mean±SD	2.0±0.9	1.7±0.7	0.66
<b>C4d deposition (C4d)</b>			
% with a C4d score>0	9 (23.7%)	3 (15.0%)	0.52
C4d score, mean±SD	0.5±1.1	0.5±0.8	0.98
<b>Interstitial infiltrates (i)</b>			
% with an i score>0	21 (55.3%)	2 (10.0%)	0.0008
i score, mean±SD	0.9±1.0	0.1±0.3	0.003
<b>Tubulitis (t)</b>			
% with a t score>0	14 (36.8%)	14 (70.0%)	0.03
t score, mean±SD	1.1±1.1	0.5±0.7	0.02
<b>TCMR diagnostic criteria, n (%)</b>	8 (21.1%)	2 (10.0%)	0.18
IA, n (%)	3 (8.8%)	2 (10.0%)	0.29
IB, n (%)	3 (8.8%)	0 (0%)	0.27
IIA, n (%)	0 (0%)	0 (0%)	1.00
IIB, n (%)	1 (2.6%)	0 (0%)	1.00
III, n (%)	1 (2.6%)	0 (0%)	1.00
<b>Vasculitis (v)</b>			
% with a v score>0	23 (60.5%)	3 (15.0%)	0.0003
v score, mean±SD	1.3±1.1	0.3±0.8	0.0003
<b>Interstitial hemorrhages, n (%)</b>	12 (31.6)	3 (15.0)	0.22
<b>Thrombotic microangiopathy, n (%)</b>	6 (15.8)	0 (0.0)	0.08
<b>Allograft glomerulopathy (cg)</b>			
% with a cg score>0	0 (0.0%)	0 (0.0%)	1.00
cg score, mean±SD	0.0±0.0	0.0±0.0	1.00
<b>Mesangial expansion (mm)</b>			
% with an mm score>0	2 (5.3%)	0 (0.0%)	0.54
mm score, mean±SD	0.1±0.4	0.0±0.0	0.59
<b>Interstitial fibrosis (ci)</b>			
% with a ci score>0	4 (10.5%)	4 (20.0%)	0.43
ci score, mean±SD	0.2±0.7	0.3±0.6	0.97
<b>Tubular atrophy (ct)</b>			
% with a ct score>0	4 (10.5%)	4 (20.0%)	0.42
ct score, mean±SD	0.2±0.7	0.2±0.4	0.80
<b>Chronic vascular changes (cv)</b>			
% with a cv score>0	16 (42.1%)	13 (65.0%)	0.16
cv score, mean±SD	1.0±1.1	0.9±1.1	0.87
<b>Arteriolar hyalinosis (ah)</b>			
% with an ah score>0	15 (39.5%)	11 (55.5%)	0.28
ah score, mean±SD	0.8±0.9	0.8±1.1	0.59



**Table 3:** Top 20 immunogenic antigens in patients with AMVR of 857 candidate antigens overexpressed in microvascular ECs

Gene ENS	Symbol	Delta Expression in Micro ECs vs Macro ECs	Protein Locus	Description	Frequency in Stable Patients	Frequency in Patients with AMVR	Intensity in Stable Patients	Intensity in Patients with AMVR	Overall Score	P Value
ENSG00000162078	ZG16B	148.5	NM_145252.1	zymogen granule protein 16B	33.33%	90.91%	2.11	2.92	87.2	0.0004
ENSG00000163431	LMOD1	144.8	BC001755.1	leiomodlin 1	25.00%	68.18%	1.04	1.99	60.4	0.0131
ENSG00000107779	BMPRI1A	1782.7	NM_004329	bone morphogenetic protein receptor, type IA	16.67%	72.73%	0.87	1.03	57.4	0.0011
ENSG00000197971	MBP	4251.9	NM_001025100.1	myelin basic protein	25.00%	63.64%	1.11	2.16	56.4	0.0251
ENSG00000169188	APEX2	23.7	NM_014481.2	APEX nuclease 2	25.00%	77.27%	0.93	1.09	55.0	0.0027
ENSG00000106789	CORO2A	433	NM_052820.1	coronin, actin binding protein, 2A	33.33%	63.64%	1.01	2.34	51.1	0.1810
ENSG00000183287	CCBE1	15174.7	BC046645.1	collagen and calcium binding EGF domains 1	8.33%	45.46%	0.60	1.59	46.0	0.0621
ENSG00000145242	EPHA5	1609.2	PV3359	EPH receptor A5	25.00%	63.64%	0.90	1.23	44.0	0.0771
ENSG00000106829	TLE4	1281.9	BC059405.1	transducin-like enhancer of split 4	33.33%	68.18%	2.05	1.99	43.5	0.0451
ENSG00000142459	EVI5L	1270.2	NM_145245.1	ecotropic viral integration site 5-like	25.00%	59.09%	0.84	1.33	41.4	0.1225
ENSG00000107679	PLEKHA1	2978.9	NM_001001974.1	pleckstrin homology domain containing, family A1	16.67%	45.46%	0.69	1.71	39.7	0.0621
ENSG00000198959	TGM2	28594.6	BC003551.1	transglutaminase 2	8.33%	45.46%	0.68	0.96	37.6	0.0621
ENSG00000082805	ERC1	2673.4	PV3626	ELKS/RAB6-interacting/CAST family member 1	8.33%	31.82%	0.39	2.31	36.0	0.0653
ENSG00000198081	ZBTB14	285.7	NM_003409.2	zinc finger and BTB domain containing 14	33.33%	63.64%	0.94	1.25	36.0	0.1810
ENSG00000128872	TMOD2	560.4	BC036184.1	tropomodulin 2	16.67%	50.00%	0.85	1.09	35.6	0.0369
ENSG00000168175	MAPK1IP1L	3083.3	NM_144578.1	mitogen-activated protein kinase 1 interacting protein 1-like	8.33%	40.91%	0.60	1.12	35.5	0.0215

ENSG00000112561	TFEB	171.1	NM_007162.1	transcription factor EB	8.33%	40.91%	0.62	0.99	33.7	0.0215
ENSG00000123836	PFKFB2	596.2	NM_006212.1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	41.67%	68.18%	1.09	1.40	33.4	0.1183
ENSG00000106123	EPHB6	119.4	NM_004445.1	EPH receptor B6	8.33%	36.36%	0.69	1.14	30.6	0.1540
ENSG00000240694	PNMA2	684.4	XM_376764.2	paraneoplastic Ma antigen 2	8.33%	36.36%	0.65	1.10	30.3	0.0381

\*Intensity represents the average ratio of observed reactivity exceeding the cut-off for sera from stable patients and patients with AMVR.

† The score was calculated using the equation  $(\text{FreqAMVR} \times \sqrt[3]{\text{IntensityAMVR}}) - (\text{FreqStable} \times \sqrt[3]{\text{IntensityStable}})$  described by Gnjatic et al<sup>7</sup>.

## FIGURE LEGENDS

**Figure 1: Study design and workflow.** A nationwide survey identified patients suspected having of early (<3 months post-transplant) microvascular (g+ptc score $\geq$ 3 according to the Banff classification) rejections of a renal allograft. After centralized Luminex<sup>®</sup> SAFB assay testing and central reading of the biopsies, 38 patients were retained for two parallel substudies. A case-control histological study (Study #1) addressed the histological characteristics of the 38 acute microvascular rejections compared to 20 patients with early acute antibody-mediated rejection associated with anti-HLA donor-specific antibodies. A case-control biological study (Study #2) focused on identifying non-HLA antibodies using several approaches and used pretransplant serum samples from unsensitized KTRs who remained stable during the first year after transplant and were used as controls. Finally, an integrated analysis of transcriptomic and proteomic data was performed to identify antibodies targeting glomerular cell-specific antigens (Study #3). For this aim, the differential transcriptomic profiles of microvascular glomerular ECs and macrovascular ECs were combined with the global seroreactivity toward protein arrays of serum samples collected immediately before transplantation from KTRs with AMVR or stable KTRs.

**Figure 2: Representative pathological characteristics of the early AMVRs. (A.)** Mean ( $\pm$ SEM) values of the elementary lesions assessed using the Banff classification in the biopsy samples from 38 KTRs at time of acute microvascular rejection. **(B.)** The g+ptc scores of the 38 individual patients with AMVR. **(C.)** Image of Periodic acid–Schiff (PAS) staining showing severe glomerulitis with partial to complete occlusion of glomerular capillaries by infiltrating leukocytes (mononuclear cells and neutrophils cells) (arrow). **(D.)** Image of PAS staining

showing severe peritubular capillaritis (ptc3) with more than 10 inflammatory cells in dilated capillaries (arrow) associated with diffuse interstitial edema (+). **(E.)** Image of PAS staining showing intimal arteritis v2 with mononuclear cells underneath the endothelium and occlusion of more than 25% of the arterial lumen (★) associated with peritubular capillaritis (+) and sparse inflammatory cells within the interstitium. **(F.)** Image of Masson's trichrome staining showing severe glomerulitis with complete occlusion of glomerular capillaries by infiltrating leukocytes and EC enlargement. EC enlargement is also present in arterioles (arrow). **(G.)** Image of Masson's trichrome staining showing thrombotic microangiopathy characterized by thrombi in the glomerular capillaries (★) associated with glomerulitis, peritubular capillaritis and diffuse interstitial hemorrhage (+). **(H.)** Image of Masson's trichrome staining showing a mixed rejection with diffuse interstitial inflammation and tubulitis (arrow), glomerulitis ⊕, peritubular capillaritis (+), arteriolitis (★) and interstitial hemorrhage.

**Figure 3: Assessment of known AECAs. (A.)** Titers of anti-AT-1R and anti-ETAR antibodies in serum samples collected on the day of transplantation from 23 patients with early AMVR without anti-HLA donor-specific antibodies and 10 nonsensitized KTRs who did not experience any rejection during their first year after transplant and were used as controls. P values were determined using the Mann-Whitney test. **(B.)** Assessments of natural polyreactive antibodies were conducted using flow cytometry to detect reactivity to apoptotic cells or using a dissociation-enhanced lanthanide fluoroimmunoassay (DELFI) to detect reactivity to malondialdehyde (MDA) in 19 patients with AMVR and 8 controls. P

values were determined using the Mann Whitney test. **(C.)** Correlation between anti-AT-1R and anti-ETAR antibody titers at the time of transplantation. **(D.)** Correlation between NABs reactive to MDA and anti-ETAR antibodies at the time of transplantation. **(E.)** Correlation between NABs reactive to MDA and anti-AT-1R antibodies at the time of transplantation. **(F.)** Analysis of the seroreactivity of serum samples from 10 stable patients and 23 patients with AMVR toward 62 non-HLA antigens using single-antigen flow bead assays. The color of each box indicates the MFI of the reaction of the sample to an individual antigen. The thresholds for defining a positive reaction of the patients with to each individual antigen were calculated based on the mean MFI of the control group of stable patients. Samples with an MFI less than the mean+3 standard deviations (SD) were classified as negative and samples with an MFI greater than the mean+3 SD were classified as positive. The number of positive samples is provided on the right and the samples that reached the threshold for positivity are indicated with a cross.

**Figure 4: EC crossmatch assays.** Sera (diluted 1:4) were incubated with ECs. Antibody binding was detected using fluorescently labeled anti-human IgG, and the MFI was measured using flow cytometry. **(A.)** Comparison of the reactivity of sera from healthy volunteers (HV, n=6) and KTRs with (n=19) or without (n=10) early AMVR and without anti-HLA DSAs toward unstimulated immortalized human glomerular CiGEnCs. The data are presented as a fold increase in the MFI compared to a pool of AB serum samples used as negative control. The P value was calculated using the Kruskal–Wallis test. Asterisks depict significant differences in pairwise group comparisons calculated using Dunn’s post test. \*\*\*P<0.01 and \*\*\*\*P<0.001. **(B.)** Sera (diluted 1:4) collected on the day of transplantation or at rejection from 4 patients with AMVR without anti-HLA DSAs were incubated with

unstimulated microvascular CiGenCs ~~ECs~~. Representative histograms showing IgG binding are shown; values indicate the geometric MFIs. **(C.)** Serial dilutions of sera from patient AMVR#11 or a pool of healthy volunteers were incubated with renal microvascular CiGenCs before the detection of antibody binding using anti-human IgG. Data are presented as the geometric MFIs. **(D and E.)** Sera (diluted 1:4) collected on the day of transplantation from 19 patients with AMVR were incubated with renal microvascular CiGenCs **(D.)** or primary cultures of macrovascular arterial ECs **(E.)** before (unstimulated) or after a 48-h stimulation with TNF $\alpha$  and IFN $\gamma$ . A pool of AB sera was used as a negative control (CTL). A cut-off of a 2-fold increase in the geometric mean value of patients' sera compared with the negative control was established to define reactive sera. **(F.)** Sera (diluted 1:4) collected on the day of transplantation from 2 patients with early AMVR or a pool of serum samples from healthy volunteers (HV, n=6) were incubated with renal microvascular ECs or epithelial cells. Microvascular ECs were used before or after *in vitro* differentiation. Representative histograms showing IgG binding are shown, and the values indicate the geometric MFIs.

### Figure 5: Integrated RNAseq-protein array analysis

**(A.)** Unsupervised PCA of the global seroreactivity profiles of serum samples collected immediately before transplantation from patients with AMVR (n=20) and stable KTRs (n=10). Average fixation signals of the immunogenic antigens were used. Ellipses of confidence (0.95) are presented for each group. **(B.)** Clustering and heat map representations of the transcriptomic data from microvascular and macrovascular ECs. Cell samples (n=3 for microvascular ECs and n=5 for macrovascular ECs) are arranged along the x-axis, whereas differentially expressed genes (n=3427) are arranged along the y-axis. The color of each cell reflects the fold change in the expression of each gene. **(C.)** Heat map representation of the

seroreactivity patterns of patients with AMVR and stable KTRs. Sera are arranged along the x-axis, whereas immunogenic antigens are arranged along the y-axis. The color of each cell reflects the normalized average fixation signal of an individual serum to one antigen.